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Antimicrobial Activity of *Lactobacillus acidophilus* Isolated from Breast Milk In Vitro

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Abstract

Probiotics are used as a method to prevent many disorders and treat diseases. *Lactobacillus acidophilus* was isolated from breast milk with the intention of studying their properties as probiotics in view of their importance isolates to simulate gastric juice antibacterial activity of bacteria isolates against enteric pathogenic bacteria. Evaluation of five positive samples out of 102 have been isolated and identified, by using selective media (MRS-VA), biochemical tests and molecular diagnosis using the 16S rRNA gene. The probiotic potential was assessed by determining antibiotic susceptibility through the disc diffusion method. Among five isolates, *lactobacillus acidophilus* was also highly resistant to simulated gastric environment, though there was a decreasing by 0.287 after incubation for three hours at 37 ºC in the simulated gastric juice with pH 2.5. *Lactobacillus acidophilus* exhibited a strong antimicrobial effect against the selected gram-negative *salmonella typhimurium*, and *E. coli*. Also, gram-positive *staphylococcus aureus* pathogens. The very small p-values (p = 0.001**). Based on findings, isolated *lactobacillus acidophilus* displayed promising probiotic characteristics, highlighting human milk as a potential source of these beneficial bacteria.

Keywords: *Lactobacillus acidophilus*, gastric juice, antibacterial activity, and breast milk.

1. Introduction

The term “probiotic” was first used in 1965, by Lilly and Stillwell, to describe substances secreted by one organism which stimulate the growth of another [1]. The use of antibiotics, immunosuppressive therapy and irradiation, amongst other means of treatment, may cause alterations in the composition and influence the gastrointestinal tract flora. Therefore, the

introduction of beneficial bacterial species to gastrointestinal tract may be a very attractive option to re-establish the microbial equilibrium and prevent disease [1].

Probiotic bacteria possess the ability to inhibit pathogenic bacteria through mechanisms such as lowering luminal pH, preventing bacterial adherence and translocation, or by producing antibacterial agents and defenses. One mechanism through which the gut microbiota prevents colonization by pathogenic bacteria involves the establishment of a physiologically restrictive environment characterized by a high concentration of acetic acid, thereby reducing luminal pH Probiotics can contribute to the establishment of beneficial bacterial communities within the intestines while effectively excluding pathogenic microorganisms [2].

Lactobacillus acidophilus is a Gram-positive, non-spore-forming, rod-shaped bacterium that belongs to the family *Lactobacillaceae*. It is a homofermentative lactic acid bacterium that primarily produces lactic acid from carbohydrate metabolism. This species is facultative anaerobic and acid-tolerant, allowing it to survive and colonize the gastrointestinal tract and other acidic niches [3]. *Lactobacillus acidophilus* is an acid-tolerant lactic acid bacterium commonly found in the human gastrointestinal tract, mouth, and vagina, as well as in fermented dairy products. It is widely used as a probiotic due to its role in improving gut health, lactose metabolism, and microbial balance [4].

L. acidophilus has the potential to decrease serum cholesterol levels, stabilize enteric microbiota, and combat cancer cells [5]. Additionally, *L. acidophilus* can inhibit the proliferation of pathogenic bacteria within the intestine, regulate the balance of intestinal flora, and enhance resistance *L. acidophilus* can initiate anti-inflammatory responses, enhance phagocytosis, induce defensing production, and modulate intestinal permeability, all of which are essential for immunity enhancement and overall gut health. The health-promoting effects of *L. acidophilus* on disease prevention, immunity enhancement, gut wellness.

The primary aim of this study is tolerance of bacteria isolates to simulate gastric juice. Evaluation of the antibacterial activity of bacteria isolates against enteric pathogenic bacteria, which include *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium*.

2. Materials and Methods

2. 1 Samples Collection

One hundred and two breast milk samples were randomly acquired from breastfeeding mothers at Al-Kut Hospital and Al-Zahraa Teaching Hospital in Wasit province, between the 1st of October 2023 and the 30th of February 2024. Cleaning process involved application of 70% ethanol alcohol on the nipple and mammary areola, with the initial drops discarded to prevent alcohol contamination. Sterile breast pumps were employed to collect milk samples, and approximately 5 to15 mL of each sample was transferred to a sterile tube. These tubes, containing samples, were then carefully packed in insulated boxes with dry ice and dispatched to the laboratory within an hour.

L. acidophilus was isolated by adding 1 mL of milk samples to 9 mL of sterilized de Man Rogosa Sharp (MRS) broth (Hi-Media, Mumbai, India). Thoroughly mix the solution and incubate it for 24 hours. The identity of these isolates was confirmed based on biochemical tests and polymerase chain reaction (PCR) analysis.

2. 2 Ethical Approvals

This study was performed in compliance with ethical standards of the College of Science, Wasit University, Al-Kut Hospital, and Al-Zahraa Teaching Hospital. Healthy lactating mothers were recruited to sampling breast milk, with their consent. No samples were kept for beyond research, and participants' personal privacy was always ensured.

2. 3 Gastric Juice Tolerance

Fresh bacterial cultures grown on MRS agar were inoculated into MRS broth and incubated at 37 °C for sixteen hours. Cultures were then harvested by centrifugation at 6000 rpm for 20 minutes, washed twice with sterile saline solution (0.85%, pH 7.0), and re-suspended in the same solution. Serial dilutions were prepared to obtain a final concentration of 10^9 CFU/mL.

Subsequently, 0.5 mL of the bacterial suspension was mixed with 4.5 mL of simulated gastric juice containing pepsin (0.3% w/v) and NaCl (0.5% w/v), adjusted to pH 2.5. The mixture was incubated at 37 °C for three hours. Viable cell counts were determined by plating serial dilutions on MRS agar using the pour-plate method, followed by incubation at 37 °C for 48 hours. Survival rates were expressed as $\log_{10}$ CFU/mL. All experiments were performed in triplicate, and mean values were calculated from 0 to 3 hours [6].

2. 4 Preparation of Enteric Pathogenic Bacteria

Pathogenic bacterial strains, encompassing *S. typhimurium*, *E. coli*, and *S. aureus*, were obtained from the Microbiology Laboratory, College of Science, Wasit University and preserved in the stock culture collection at 4 °C in a 20% glycerol solution. Prior to their application in the appropriate medium, *S. aureus*, *E. coli*, and *Salmonella typhimurium*, underwent sub culturing on Mannitol salt agar and MacConkey agar, respectively [7].

2. 5 Antimicrobial Activity

Antimicrobial activity was carried out according to the agar well diffusion method. *E. coli*, *S. aureus*, and *S. typhimurium* were cultured in nutrient broth for 24 hours. Then, microbial density was adjusted to 107 CFU/mL by a spectrophotometer at a wavelength of 625 nm, and it was found that the turbidity (absorbance) was 0.08 to 0.1. The nutrient agar plate surface is inoculated by spreading a volume of pathogenic bacteria *E. coli*, *S. aureus*, and *S. typhimurium*.

Meanwhile, Cell-free culture supernatants (CFCS) of *L. acidophilus* isolates were grown in MRS broth for 24 hour and obtained by centrifuging the culture broth at 5000 rpm for 30 minutes at 5° C. 100 µL of the CFCS was placed into the wells of the nutrient agar that are 7 mm in diameter and aseptically perforated with a sterile tip, and the nutrient agar plates were incubated at 37 °C between 14 and 15 hours. Finally, the diameter of the clear zones around each well was measured [8].

2. 6 Statistical Analysis

Data was analyzed with the help of Statistical Package for Social Science (SPSS). The data were represented by mean, standard deviation (SD) and standard error (SE). The influence of duration of incubation on the survival of *Lactobacillus acidophilus* in simulated gastric juice was assessed by paired t-test. There were significant differences in the antimicrobial activity against pathogenic bacteria tested as determined by one-way analysis of variance (ANOVA). P values ≤ 0.05 were considered significant [9].

3. Results and Discussion

3. 1 Tolerance of *Lactobacillus acidophilus* isolated to gastric juice simulated

Tolerance toward acidic conditions is recognized as one of the important factors for probiotic bacteria to play their role, since

probiotic bacteria may encounter low pH both in the process of food manufacturing and when passing through the stomach. Thus, tolerance of *Lactobacillus acidophilus* to low pH was studied as a model for assessing their ability to survive in acidic environment. Bacteria recovery was assessed following various incubation times, and the results were expressed as means with their corresponding standard deviation (SD) as well as standard error (SE). Statistical analysis was conducted in order to point out the impact of incubation time on bacterial survival, and thus further describing stability and resistance of analyzed strains against acid stress.

Table 1: The average acidity tolerance of L. acidophilus at zero and three hours.

Sample	Time / hours	Mean (log)	SD	SE	P. value
1	0	5.312	0.0105	0.0061	0.002**
1	3	5.116	0.0040	0.0023	
2	0	5.256	0.0135	0.0078	0.001**
2	3	4.946	0.0144	0.0083	
3	0	5.299	0.0174	0.0100	0.001**
3	3	5.004	0.0075	0.0043	
4	0	5.111	0.0137	0.0079	0.001**
4	3	4.768	0.0242	0.0140	
5	0	5.269	0.0123	0.0071	0.001**
5	3	4.977	0.0230	0.0133	

Table 2: Summary acidity tolerance of L. acidophilus at zero and three hours.

Parameters	Mean	SD	SE	P. value
Before incubation (zero time)	5.249	0.0751	0.0194	0.001**
After incubation (three hours)	4.962	0.118	0.030	

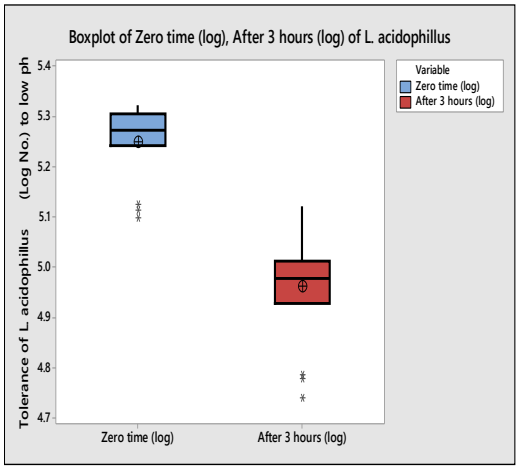

Figure 1: Box Plot Showing Survival Distribution of Lactobacillus acidophilus Under Simulated Gastric Juice.

Table one, and table two, viable cell count was significantly reduced due to incubation at low pH following simulated gastric juice treatment, *Lactobacillus acidophilus* was able to survive the simulated gastric juice challenge. The most notable aspect was that there was no loss of viability (there were viable counts above 4 log CFU/mL) after acid exposure for neither species. Such a survival level generally is recognized as being enough for probiotic activity and successful passage through the

gastro-intestinal tract, suggesting that the two strains are naturally resistant to gastric acidity.

The decrease that was observed in viable cell number recorded in this study is a normal physiological response of lactic acid bacteria to acidic stress and does not represent serious cellular injury. Likewise, *L. acidophilus* was also highly resistant to simulated gastric environment, though there was a decreasing trend in log count values significantly with advancing time. Acid tolerance in *L. acidophilus* was known and regarded as the characteristic of this species. This resistance is mainly due to the induction of acid shock proteins that protect key cellular components from denaturation via acid and adaptive changes in fatty acid composition of the membrane.

Especially, an increase in the proportion of unsaturated fatty acids can improve membrane fluidity and decrease proton permeability to alleviate proton influx and promote bacterial resistance to acid stress [10-12]. Besides the membrane-associated adaptations, intracellular metabolic changes contribute to acid tolerance. Intracellular pH homeostasis is maintained through malolactic fermentation which converts malic acid to lactic acid and consumes protons, thereby minimizing cytoplasmic acidification at low pH [13]. Moreover, fermentable carbohydrate sources have also a role in the provision of ATP to LAB and maintenance of activity of proton pumps, which is reduced by rapid. The box plot figure illustrates the distribution and variability of bacterial survival response to acid stress. Furthermore, no extreme outlier could be observed in the box plots, which is also further confirmation of the high quality of experiment data showing little replicates fluctuation.

Slight discrepancies from the distributions are probably due to natural biological diversity instead of technical bias. Results of the current study are well correlated with other studies that have been published on acid tolerance among probiotic Lactobacillus. Several investigations have indicated that probiotic strains are able to withstand reductions in terms of viable cell counts which, although statistically significant, are biologically acceptable after exposure to simulated gastric juice and the minimum values are still higher than 4 log CFU mL −1 [14, 15].

The acid tolerance of *L. acidophilus* in the present study is also in agreement with previous reports that observed their survival at simulated gastric conditions with minimum loss in viability [12,10]. Found that

viable counts were also reduced similarly at acidic conditions and ascribed this tolerance to the existence of conserved stress responses chained to adaptation such as those controlling acid shock protein expression and remodeling of membrane. The general similarity in the patterns of response incorporated in this study, compared with other studies, indicates that they reflect genuine differences in strains rather than artifacts resulting from methodology or subjectivity, and hence are within the range expected of well-defined probiotic strains.

3. 2 Antimicrobial Actions of *Lactobacillus acidophilus* on Selected Pathogens

The in vitro antimicrobial activity of *Lactobacillus acidophilus* was studied towards three human pathogens. Findings demonstrated that *Lactobacilli* used had an inhibition effect on all tested pathogens. It can be observed that *L. acidophilus* against *Salmonella spp.*, 15.933 ± 2.017 mm and 21.067 ±17.51 mm showed the mean zones of inhibition while *Staphylococcus spp*., was recorded with a mean of an inhibition zone measuring as listed in table 3, where statistical analysis showed significant differences (p = 0.001**).

Table 3: Mean value of antimicrobial activity of *L. acidophilus* against *Salmonella*, *E. coli* and *Staph*.

	Salmonella		*E. coli*		*Staph.*	
	Mean mm	SD	Mean mm	SD	Mean mm	SD
L.acidophilus	17.200	2.042	15.933	2.017	21.067	17.51
P. value	0.001 **					

Data in table three indicates that *Lactobacillus acidophilus* exhibited a strong antimicrobial effect against the selected gram-negative (*Salmonella spp*., and *E. coli*) and gram positive (*Staphylococcus spp*.) pathogens. The very small p-values (p = 0.001**) indicate that the observed inhibitions are biologically relevant and not due to randomness.

The amount of standard deviation observed, relatively speaking, in the case of *L. acidophilus* against *Staphylococcus spp*. suggests that antimicrobial activity may be subject to some variability depending on metabolite production, incubation parameters or strain heterogeneity. Comparably high variability has been described in other studies highlighting the need of standardization of assay conditions [16]. Reported zones of inhibition between 15 and 22 mm for *L. acidophilus* against *E. coli* as well as *Staphylococcus aureus* [17].

S. typhimurium results obtained here confirm the antimicrobial activity of *L. acidophilus*, broad-spectrum effects,

indicating their potential use as probiotic strains and bio preservatives in foods and therapy. Additional in vivo investigations are suggested to confirm these in vitro results and to study potential synergy with other probiotic microorganisms.

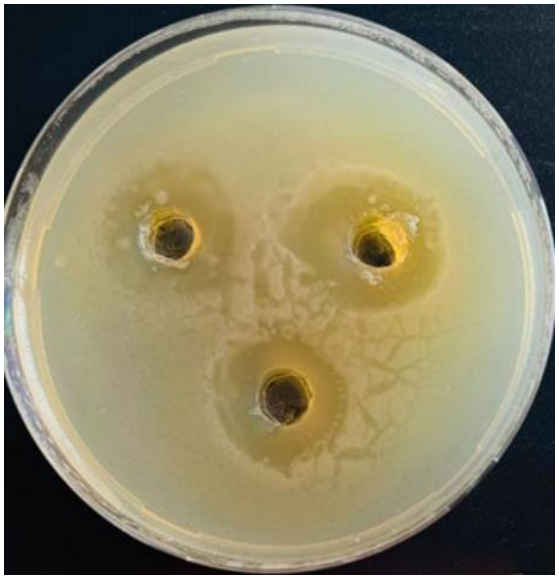

Figure 2: Inhibition zones of *L. acidophillus*, against *E. coli*.

4. Conclusion

The study shows that *Lactobacillus acidophilus* may be safely isolated from human breast milk and possesses key probiotic features. Tested strains appeared to have a good survival capacity in simulated gastric juice, keeping the number of viable cells higher than that necessary for probiotic activity after three hours of incubation.

This suggests that *L. acidophilus* is well adapted to acidic stress that enables it to transit the GI tract. *Lactobacillus acidophilus* showed a pronounced inhibition against some pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhimurium*. This is verified by the presence of inhibition zones for both gram-negative and gram-positive pathogens, indicating their possible utilization in controlling enteric infection.

Therefore, human breast milk may be considered as a natural source of probiotic *L. acidophilus*. However, additional in vivo research is suggested to further verify the probiotic properties of these isolates whether manufacturer for food or medicinal purposes.

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